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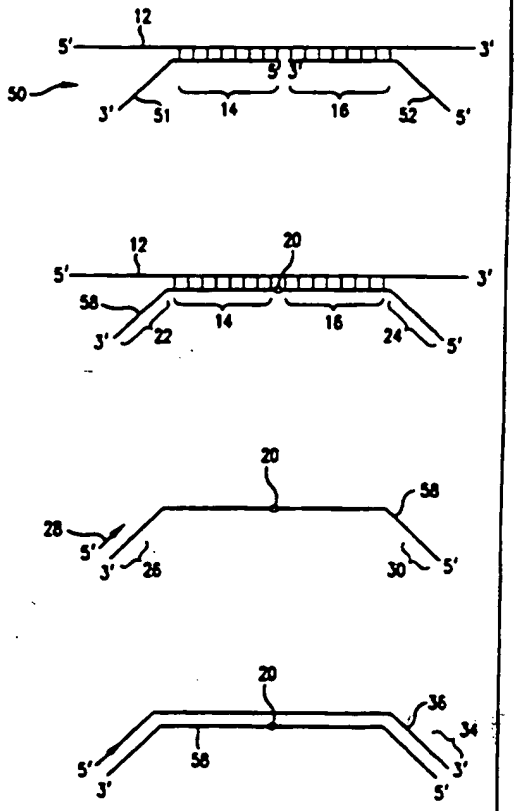
(54) Title: MULTIPLEX LIGATIONS-DEPENDENT AMPLIFICATION

(57) Abstract

A method of multiplex amplification features a plurality of split probe reagents ("SPRs") each of which includes a target specific region defined by its 3' and 5' ends and, in non-complementary regions ("NCRs"), primer binding sites ("PBSs") that are common to each split probe reagent. The 3' and 5' ends of each SPR are ligated together only when hybridized to its target-specific template strand but, once joined, all SPRs can be amplified by a common primer set in a PCR reaction. SPRs may be a continuous strand, the ends of which are ligatable to form a loop, or they may be distinct polynucleotide pairs. Specialized sequence segments may be employed to facilitate detection on the basis of specific sequences and/or length.



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# Multiplex Ligations-dependent Amplification

## Field of the Invention

The invention relates to methods for amplifying and detecting a target nucleic acid sequence and, more particularly, to a method for specifically amplifying multiple target sequences using a single pair of primers.

## Background of the Invention

One well-known method for amplification of target nucleic acids is the polymerase chain reaction (PCR). In PCR two primers (of opposite "sense") are employed in excess to hybridize at the outside ends of complementary strands of the target nucleic acid. The primers are each extended by a polymerase using the target nucleic acid as a template. The extension products are dissociated from the original target strand and the extension product of one primer becomes template for extension of the other primer as is well understood in the art. The cycle of dissociation, reannealing and extension is repeated to increase geometrically the number of target sequence molecules. PCR is described further in U.S. Patents 4,683,195 and 4,683,202, both incorporated herein by reference.

An alternate mechanism for amplification of target nucleic acids is known as ligase chain reaction (LCR). In LCR, two sets of probe partners are used which includes one set of primary probes (first and second probe partners) and a second set of secondary probes (third and fourth probe partners) all of which are employed in excess. One probe partner hybridizes to a first segment of the target strand and the other probe partner hybridizes to a second segment of the same target strand, the first and second segments being contiguous (either with or without a template-dependent correction step) so that the primary probes abut one another in 5′ phosphate-3′ hydroxyl relationship and so that a ligase enzyme or other reagent can covalently fuse or ligate the two probes of the partner set into a fused product. In addition, a third (secondary) probe can hybridize to a portion of the first probe and a fourth (secondary) probe can hybridize to a portion of the second probe in a similar abutting fashion, either with or without correction. Of course, if the target is initially double stranded, the secondary probes will also hybridize to the target complement in the first instance. Once the fused strand of primary probes is separated from the target strand, it will hybridize with the third and fourth

probes which can be ligated to form a complementary, secondary fused product. The fused products are functionally equivalent to either the target or its complement. By repeated cycles of hybridization and ligation, amplification of the target sequence is achieved. This technique is described more completely in K. Backman, *et al.* EP-A-0 320 308 and in EP-A-0 439 182, both incorporated by reference in their entirety.

A detection method known as oligonucleotide ligation assay ("OLA") is described in U.S. Patent 4,883,750 (Whiteley). This process involves detection of a target strand using a diagnostic probe and a contiguous probe, the two of which are ligated by a ligase in the presence of a target-template. In a variation of this, a gap between the two probes is first filled by a polymerase extension before ligation. See U.S. Patent 5,185,243 (Ullman, et al) and EP-A-0 246 864 (Carr).

Another nucleic acid hybridization detection method is disclosed in EP A 0 128 332 (Enzo). According to this method a circular "bridging molecule" (polynucleotide) is used to hybridize with both a target analyte and to a polynucleotide containing a label or complexing moiety. The complexing moiety is used to attach a label or reporter group to the target strand, via the bridging molecule.

Other amplification methods include the so-called "rolling circle" method, which is disclosed in WO92/01813 (Ruth and Driver) and elsewhere. In this method a circular, single-stranded template is formed and a primer hybridizes to it. The primer is extended around the circle multiple times, thereby creating multiple copies complementary to the circular template. Preferably the circular template includes the complement of a cleavage site so that the resulting repeating polymer can be cleaved into segments representing one circumference.

PCR has been used in a multiplex manner to determine the presence of multiple target sequences in a single reaction. EP-A-0 364 255 describes the use of multiple primer sets to simultaneously amplify multiple DMD target sequences by PCR. A similar disclosure is made in Chamberlain, et al., *Nucl. Acids Res.*, 16:1141-56 (1988). LCR has similarly been used in a multiplex manner to determine the presence of multiple target sequences in a single reaction (see, e.g. WO93/20227). In both cases, a distinct primer or probe set is required for each target sequence desired to be detected. Since all primer/probe sets are present in the same reaction mixture, it follows that amplification conditions must be adjusted so that signal from each primer/probe set develops at approximately the same time. In other words, it is not possible in a

multiplex reaction to optimize signal development by changing cycle parameters (e.g. cycle time, cycle temperature or the number of cycles) from one primer/probe set to another; all must amplify within a constant set of cycle parameters. This has necessitated careful titration of primer/probe concentrations and has resulted in compromises that reduce the overall efficiency of amplification.

The generation of circularized probes following a target specific ligation step has been described by Nilsson et al. in *Science*, 256:2085-2088 (1994). The circularized probes, dubbed "padlock probes" due to their catenation with the target, were used for the identification of clones in genomic libraries, fragments in blots of whole genomes or for in-situ analysis of chromosomes. The ability to perform direct hybridization assays with reduced background is possible by removal of label from unligated probes, either by alkaline phosphatase cleaveage of $^{32}P$ label, or by exonucleolytic degradation of the unhybridized oligonucleotide probes. Circularized probes are resistant to label removal and degradation since they contain no free 5' or 3' ends.

Summary of the Invention

The present invention overcomes these and other difficulties by providing methods and kits for multiplex amplification in which a single common primer pair is used to amplify split probe reagents specific for each target sequence. Thus, a single primer set is used for amplification and the reaction conditions can be optimized for this single primer set to maximize the efficiency of amplification.

In a first aspect, the invention is a method for amplifying a target nucleic acid sequence comprising:

(a) forming a reaction mixture under hybridizing conditions with

(i) a sample suspected of containing a target strand with a target sequence of interest, the target strand being present in single stranded form;

(ii) at least one split probe reagent having a 5′ end complementary to a first segment of the target strand and a 3′ end complementary to a second segment of the target strand, the second segment being sufficiently near the first segment such that 5′ end can be joined to the 3′ end when the split probe reagent is hybridized with the target strand, wherein said 5′ end and said 3′ end are on two distinct polynucleotides or on different ends of one continuous polynucleotide; the split probe reagent further having a first non-complementary region located downstream of the 5′ end and not complementary to the target strand, and a second non-complementary region located upstream of the 3′ end and not complementary to the target strand; and

(iii) an agent for ligating together said 3′ and 5′ ends of the split probe reagent;

(b) ligating together said 3′ and 5′ ends of the split probe reagent while hybridized with the target strand thereby to form a ligated probe having a ligation junction;

(c) separating the ligated probe from the target strand;

(d) treating the reaction mixture under hybridizing conditions with:

(i) an excess of primers wherein a first primer has a sequence complementary to a primer binding site ("PBS") located in said first non-complementary region; and wherein a second primer has a sequence identical to a site ("PBS′ ") located in said second non-complementary region, with the proviso that if said split probe reagent is continuous the primer binding site located in said first non-complementary region is upstream of said site located in said second non-complementary region;

(ii) a supply of deoxynucleotide triphosphates; and

(iii) an agent for inducing extension of the primers;

(e) extending the first primer to form an extension product therefrom;

(f) treating the reaction mixture under denaturing conditions to separate primer extension products from their templates;

(g) treating the reaction mixture under hybridizing conditions to anneal the primers to the ligated probe or to extension product of the first primer and extending the primers to form extension products therefrom.

In a multiplex system, there is generally one or more split probe reagents ("SPR") for each target one desires to detect. Each SPR has a 3'/5' end specific for a different particular target, and has two sequence segments common to all SPRs that serve as primer binding sites ("PBS") or complements thereof (the so-called PBS' site).

Preferably the agents for ligating and for inducing extension are enzymatic agents such as a DNA ligase and DNA polymerase, respectively. The polymerase is preferably thermostable. Since the ligase need not be active through the cycling steps, it generally need not be thermostable, but in cases where hybridization is performed under stringent conditions, (e.g. high tempreature) some degree of thermostability may be desirable. Generally steps (f) and (g) are repeated from 2 to 100 times, more typically from 15 to 50 times.

It may be preferable to include specialized segments for detection within the SPR in a location that will be amplified by the primers. These segments may include another sequence that is common for each SPR/target, specific for each SPR/target, or sequences located between the PBSs that vary sufficiently in length that amplification products from one SPR can be distinguished from amplification products from a different SPR on the basis of length differences.

It may be desirable to separate unligated SPR from the reaction mixture either before or after the ligating step, but prior to amplification. This may be accomplished by removal of the SPR:target complexes or, in one embodiment, by degradation of excess single stranded SPRs.

In another aspect, the invention is a kit containing the reagents necessary to perform the above methods, and optionally including instructions for use. The reagents may be in one or more containers and will include at least:

(a) one least one split probe reagent as defined above for each target to be detected;

(b) an agent for ligating the split probe reagent;

(c) primers as defined above;
(d) a supply of deoxynucleotide triphosphates; and
(e) an agent for inducing extension of the primers.

Brief Description of the Drawings

Figures 1a through 1h are a representation of the method steps of amplifying a target strand of nucleic acid according to one embodiment of the present invention, wherein the split probe reagent comprises one continuous polynucleotide, the ends of which are complementary to adjacent segments of the target sequence such that the ends are ligatable when hybridized to the target.

Figures 2a through 2h represent the method steps of the embodiment of shown in Figures 1a to 1h wherein there is no target sequence present.

Figures 3a through 3h are a representation of the method steps of amplifying a target strand of nucleic acid according to a different embodiment of the present invention, wherein the split probe comprises two distinct polynucleotides, the ends of which are complementary to adjacent segments of the target sequence such that the ends are ligatable when hybridized to the target.

Figures 4a through 4h represent the method steps of the embodiment of shown in Figures 3a to 3h wherein there is no target sequence present.

It should be noted that the subparts (a-h) of Figures 1-4 represent the same corresponding stage of the process in each of these Figures. Also, Figures 1 and 2 are related in showing the same embodiment with and without target, respectively. Figures 3 and 4 are similarly related, showing the same embodiment with and without target, respectively.

Detailed Description of the Invention

The various aspects of the present invention will now be described in more detail. It will be noted that all publications, patents and published patent application mentioned herein are incorporated in their entirety.

*A. Terminology*

"Amplification" refers to the process by which additional copies of a target sequence are synthesized. Generally amplification involves repeated cycles of annealing, synthesis and denaturation. Synthesis is typically by a process of extension or elongation. "Multiplex" amplification refers to an amplification process wherein a plurality of distinct target sequences are amplified simultaneously in the same cycles of amplification. Plurality means at least two, preferably three or more distinct sequences.

"Split probe reagent" or "SPR" refers to a reagent that is one or two polynucleotides which include ends complementary to a specific target nucleic acid sequence. "Polynucleotide" includes non-natural binding reagents, such as peptide nucleic acid analogs (PNAs), that are capable of specifically binding to natural nucleic acids. A split probe reagent ("SPR"), whether consisting of one or two polynucleotides, generally has total length between 20 and several hundred nucleotides. More typically, the length of a single, continuous split probe reagent is between about 50 and 300 nucleotides; and the length of each half of a dual split probe reagent is between about 15 and 100 nucleotides. While the minimum length is important, the maximum length is dictated only by manufacturing economy and practicality.

Split probe reagents can be made by synthetic methods such as the phosphoramidite or H-phosphonate methods, particularly when shorter probes are desired, or they can be made by standard cloning methods. It is also possible to synthesize a split probe reagent in one or more smaller fragments and to ligate the fragments together in a desired order using one or more synthetic templates that span the assembly junctions. Alternatively, a single strand template can be synthesized and large quantities of a complementary split probe reagent can be manufactured using a polymerase and a single primer that hybridizes to the template at the 3' end.

"End" refers to a region or segment of a polynucleotide which includes the terminus and the next several adjacent nucleotides. The precise length of an "end" is relatively unimportant and variable. For example in the split probe reagent, an "end" may include up to the last 50 nucleotides of the probe, usually up to the last 30 and more typically the last 5 to 25 or so nucleotides. "End" may refer to either the 5' end or the 3' end. In the case of a split probe

reagent comprising two distinct polynucleotides (and therefore having four ends), "end" refers to those ends which are target-specific and will participate in the ligation event. By contrast, "terminus" refers to the very last 5' or 3' nucleotide of a polynucleotide.

It is not necessary that the two "ends" be the same length. In fact, if the hybridization and ligation of the SPR take place in the presence of an agent for inducing extension and a supply of nucleotide triphosphates, it may even be desirable to use a 5' end that is longer than the 3' end. This tends to facililtate hybridization of the 5' end relative to the 3' end and is important to avoid extension of the 3' end on the target under these conditions.

According to the present invention the ends of the split probe reagent ("SPR") are designed to be specific for a particular target of interest so that, when hybridized to the specific target, the ends are joinable to one another. In this regard the patent of Whiteley provides additional teaching with regard to target specific sequences that are ligated on the target-template.

"Downstream" refers to the 3' direction while "upstream" refers to the 5' direction on any given strand. Thus, when a particular sequence or event is located or occurs "downstream" of another specified location, the sequence or event can be found moving along the same strand in the 3' direction of the specified event. As a result, the downstream (as well as upstream) directions of two complementary nucleic acid strands hybridized in conventional anti-parallel fashion will be in opposite directions.

"Hybridization" or "hybridizing" conditions is defined generally as conditions which promote annealing. It is well known in the art, however, that such annealing is dependent in a rather predictable manner on several parameters, including temperature, ionic strength, probe length and G:C content of the probes. For example, lowering the temperature of the reaction promotes annealing. For any given set of probes, melt temperature, or Tm, can be estimated by any of several known methods. Typically, diagnostic applications utilize hybridization temperatures which are slightly below the melt temperature. Ionic strength or "salt" concentration also impacts the melt temperature, since small cations tend to stabilize the formation of duplexes by negating the negative charge on the phosphodiester backbone. Typical salt concentrations depend on the nature and valency of the cation but are readily understood by those skilled in the art. Similarly, high G:C content and increased probe length are also known to stabilize duplex formation because G:C pairings involve 3 hydrogen bonds where A:T pairs have just two, and because longer probes have more hydrogen bonds holding the probes together.

Thus a high G:C content and longer probe lengths impact the "hybridization conditions" by elevating the melt temperature.

Once probes are selected for a given diagnostic application, the G:C content and length will be known and can be accounted for in determining precisely what "hybridization conditions" will encompass. Since ionic strength is typically optimized for enzymatic activity, the only parameter left to vary is the temperature. For improved specificity, the hybridization temperature is selected slightly below the Tm of the probe; typically 2-15 ° C below the Tm. Thus, obtaining suitable "hybridization conditions" for a particular probe set and system is well within ordinary skill of one practicing this art.

Conversely, "denaturing" or "dissociating" conditions refer to conditions where stringency is increased such that a given probe or probes no longer hybridize. For example, under given ionic conditions increasing the temperature to a point 5-15° C above the Tm of a probe:template duplex results in a denaturing condition.

"Ligate" means to covalently join. Agents for ligating include enzymatic, chemical and photochemical means. Preferred enzymatic ligating agents include DNA ligases and the like. Thermostability is not required of these ligating agents. Suitable enzymatic ligases are commercially available from numerous sources, such as New England Biolabs, Stratagene, Molecular Biology Resources, and others. Photochemical ligation methods are also known in the art. See e.g. EP-A-0 324 616 (Amoco) and WO90/01069 (Segev). When the ends of the split probe reagent are ligated, a ligation junction is formed. The ligation junction should be formed so as not to interfere with the ligated product serving as template in subsequent cycles.

"Extend" means to grow or to make longer as is well known in the art of primer extension and polymerase chain reactions, and generally employs an agent for inducing extension and a template. Agents for inducing extension include, for example, polymerases and reverse transcriptases. As in PCR, preferably the agent for inducing extension is thermostable in order to withstand the denaturing conditions of strand separation. Numerous thermostable polymerases are commercially available, including those derived from *Thermus aquaticus* ("*Taq*") and *Thermus flavus* ("*Tfl*") organisms. Enzyme fragments, such as Klenow and Stoffel, that have the ability to extend are included within inducing agents.

"Primer" is used with its conventional meaning and refers to an oligonucleotide which can be used for a template-directed extension reaction. For example, when an oligonucleotide having a free 3' hydroxyl is hybridized

in the presence of deoxynucleotide triphosphates with a DNA template that continues in the 5' direction, it presents the proper substrate for a DNA polymerase to extend the primer by the template directed addition of nucleotide triphosphates. It is common knowledge that PCR amplification requires two primers oriented in opposing directions; however, the sequences of the primers of the invention need not be different. In other words, the same primer can prime extension in both directions in the present invention, since the PBSs need not be target specific. This may not be desirable, however, due to the potential for the PBSs to cross hydridize with one another.

"Target" refers to the section of nucleic acid that one hopes to amplify. It may comprise RNA or DNA and may or may not originate from a clinical specimen, such as blood, urine, CSF, saliva, tissue or cell scrapes, mucous, and the like. When extracted from a clinical specimen, it may be necessary to lyse a cell to release the target nucleic acid into the reaction mixture. Methods such as heating, detergents, base, and mechanical pressure are well known and suitable for this purpose. "Target" may also refer to a secondary mediator or intermediate probe that is capable of hybridization with a primary target through one or more intermediary binding members; and to any other natural or synthetic nucleic acid or analog thereof. Target strands may be present initially in double-stranded form which are separated for hybridization with SPRs.

Where "target" originates from a clinical specimen, the notion of "multiplex" may take on several nuances. In genetic testing, for example, multiplex targets can often be selected from among the several known genetic mutations that contribute to a genetic disease. This is the case in cystic fibrosis or DMD. Conversely, a multiplex assay may comprise tests for several genetic diseases in one assay. In the field of infectious diseases a similar situation holds true. One might construct a multiplex assay that tests for all types of human papillomavirus; or for all known HIV variants; or for multiple species of *Mycobacterium*. Conversely, a multiplex assay might examine multiple pathogens that are related only in that they have a propensity for infecting the same specimen types. A multiplex test for both *Chlamydia trachomatis* and *Neisseria gonorrhoeae* illustrates this type of multiplex.

Regardless of the type of multiplex assay, a particularly useful technique for which the invention is adapted is allele discrimination. Whether for detection of point mutations or determination of antibiotic resistance, the power of the present invention to discriminate alleles differing by a single base compares favorably with OLA and its gap-filling cousins described below.

Mismatches can be distinguished when they occur at or near either the 5' or 3' terminus of the split probe reagent, or in the gap if a gap-filling split probe is employed. The advantage of the present invention permits amplification by a common primer pair, but only if the desired allele was present in the first instance to ensure the ligation junction.

"Affinity member" refers to either member of a pair of specific binding ligands. An affinity member thus always has a corresponding or other member to which it will specifically bind. Many examples of such affinity members are known and include antibody-hapten pairs, complementary polynucleotide pairs, biotin-avidin pairs; sugar-lectin pairs, protein-receptor pairs, nucleic acid-nucleic acid binding protein pairs and the like.

The term "label" refers to a molecule or moiety having a property or characteristic which is capable of detection. A label may be directly detectable, as with radioisotopes, fluorophores or chemilumiphores; or a label may be indirectly detectable, as with haptens or polynucleotide tails. When indirect labels are used for detection or signalling purposes, they are used in conjunction with a signalling entity complex. A "signalling entity" is a molecule or moiety which provides the detectable property or characteristic. The signalling entity may be direct, as with a colloidal particle (e.g. colloidal gold or selenium); or it may be indirect, as with an enzyme (e.g. alkaline phosphatase, β-galactosidase or horseradish peroxidase). Indirect signalling entities may require additional components, e.g. substrate, as is well known in the art. The "signalling entity complex" includes a signalling entity conjugated to specific binding partner, such as an antibody or polynucleotide. Such conjugates may be prepared according to any known method of conjugation.

*B. Methods of the Invention*

The invention will now be described with reference to the drawing figures. Figures 1 and 2 represent one embodiment; Figures 3 and 4 represent another embodiment. Also, Figures, 1 and 3 depict what happens when target is present; Figures 2 and 4 depict what happens when target is absent. It should be noted that the subparts (a-h) of Figures 1-4 represent the same corresponding stage of the process in each of these Figures. For ease of discussion, the Figures and the ensuing description describe amplification and detection of a single target. However, the true advantage of the invention is realized when multiple targets are amplified and detected at one time using multiple split probe reagents, one specific for each target and each having in common the sequences for primer binding sites.

As mentioned previously, there are two main embodiments of the invention. Referring first to Figure 1, a split probe reagent ("SPR") 10 comprises a continuous single-stranded polynucleotide. In the other major embodiment (Figure 3), the SPR 50 comprises two distinct polynucleotides or halves 51 and 52. Since many elements are common to both embodiments, they are designated with single reference numeral in both embodiments. Where differences occur between embodiments they are separately discussed.

In a first step of the method, the split probe reagent or "SPR" is combined under hybridizing conditions with a solution suspected of containing a target nucleic acid sequence. As is shown in Figures 1a and 3a, the SPR 10, 50 includes a 5' end 14 and 3' end 16 which are complementary to and hybridize specifically with the target 12 at adjacent or nearly adjacent positions to facilitate joining of the 5' end 14 to the 3' end 16. In this regard, it is well known in the art that DNA ligases are capable of covalently joining such ends which present a 5' phosphate and a 3' hydroxyl termini at adjacent positions on a template. Other joining methods may be used when the positions are not precisely adjacent. For example, both chemical and photochemical joining methods have been described in the art (See EP-A-0 324 616 (Amoco) and WO90/01069 (Segev). In yet another variation, a small gap may be left between the 5' and 3' termini, the gap being filled prior to joining by a polymerase and a supply of less than all four nucleotide triphosphates. This type of gap filling reaction is well known in the art and is described in U.S. 5,185,243 (Ullman), EP-A-0 246 864 (Carr), and EP-A-0 439 182 (Abbott).

Once the SPR 10, 50 and the target 12 have hybridized, an agent for ligating the 3' and 5' ends is employed to form a ligated probe 18, 58 having a ligation junction 20 as shown in Figures 1b and 3b.

The SPR 10, 50 comprises several other segments which should be discussed. SPR 10 comprises one or more regions that are not complementary to the target sequence. These are described as a first non-complementary region ("NCR") 22, which is downstream of the 5' end 14, and a second non-complementary region ("NCR") 24, which is upstream of the 3' end 16 (see Figure 1b). It should be understood that in SPR 50 (and ligated probe 58) the first NCR 22 typically extends downstream all the way to the 3' terminus. Similarly, second NCR 24 extends upstream all the way to the 5' end. However, SPR 10 (and ligated probe 18) comprises but a single, continuous strand looping back on itself. In this embodiment, the first NCR 22 can extend only up to the second NCR 24 and no further. In other words, the first and second NCRs cannot overlap. It is arbitrary, however, where in the loop these

NCRs converge. To simplify the discussion, it will be presumed that the point of convergence is arbitrarily set nearer to the 5' end, i.e. in the lower left portion of the loop (see Figures) and that the 5' ends of the primer binding sites (see below) define the boundary of the NCRs. In this way, the primers amplify a regions including both entire NCRs and the 3' and 5' ends.

Referring now to Figures 1c and 3c, it will be seen that the first NCR 22 includes a first primer binding site ("PBS") 26. It is to this site that an amplification primer 28 will bind as is discussed below. In addition, second NCR 24 includes a site 30 which contains a sequence identical to that of a second primer 32 (see Figures 1e and 3e). As is well understood in the art of PCR, the site 30 is complementary to a second primer binding site or PBS 34 as shown in Figures 1d, 1e, 3d and 3e. For this reason, the site 30 may be referred to as "the second PBS' site" the "prime" designation being a common way to identify a complement.

It is important for multiplexing according to the invention that the first and second PBS be the same for each SPR. While exact sequence identity is not essential– primers can be made to hybridize and extend on templates with some mismatches– there is no reason not to utilize exact identity. Since target specificity lies in the 5' and 3' ends (14,16) of the SPR, the remainder of the polynucleotide need not have unnecessary variance. In this way a common set of primers is used to amplify the SPR. but amplification results only if the split probe has been joined to form the ligation junction.

Both the first and second NCRs 22, 24 may also contain other specialized sequences for various purposes, such as to facilitate separation and/or detection of hybrid complexes. These are discussed in more detail below.

Once a ligated probe 18, 58 is formed, it serves as a new template for amplification by a pair of common primers. Prior to amplifying this template, however, it may be preferable to separate ligated probe from unligated SPRs. This can be done by a number of techniques. For example, under hybridizing conditions the complex of ligated probe and target may be separated from the reaction mixture through the use of affinity members. Affinity members useful for this purpose include antibodies capable of recognizing duplex structures, and/or a capture hybridization probe specific for a different segment of the target strand. Alternatively, in the case of the dual SPR 50, an affinity agent attached to the polynucleotide 52 may also be used for this purpose. Although this affinity mechanism will isolate both ligated and unligated split probe halves 52, it will at least remove unligated split half 51 which is more likely to generate background than half 52.

As an alternative to physically separating ligated probe complexes, it is also possible for continuous strand SPRs to perform a separation by degrading unligated and uncomplexed single-stranded probes. Exonucleases are known to attack polynucleotides from exposed ends; but there are no exposed ends in the ligated continuous probe 18.

Whether or not complexes are separated, the next step involves the performance of PCR using the ligated probe as template. This step may involve the addition of reagents suitable for amplification or, with suitable precautions, the amplification reagents may have been present in the reaction mixture from the start. As illustrated in Figures 1c and 3c, the reaction mixture is placed under hybridizing conditions such that first primer 28 hybridizes with the first PBS 26 located in the first NCR 22. In the presence of an agent for inducing extension and suitable nucleotide triphosphates, primer 28 is extended using the ligated probe as its template to form an extension product 36. Upon denaturation, the ligated probe 18 or 58 is separated from the extension product 36 as is shown in Figures 1e, 1g, 3e and 3g. As additional cycles of denaturation, annealing and extension are performed, the excess of first primers 28 continue to hybridize to ligated probe 18, 58 and to form extension products 36 as shown in Figures 1g, 1h , 3g and 3h. It will be obvious that each extension product 36 will include a second PBS 34 as a result of extension over the second PBS' site 30 found in the second NCR. As shown in Figures 1e and 3e, second primer 32 will hybridize under hybridizing conditions to the PBS 34 and, in the presence of an agent for inducing extension and nucleotide triphosphates, will be extended to form second extension product 38. It will be readily recognized by those skilled in the art that extension product 38, by virtue of extension to the end of extension product 36, will also contain the first PBS 26. It is this inclusion of the second PBS in the extension products of the first primers, and vice versa, which permits the exponential amplification in PCR reactions.

In Figures 2a to 2h and 4a to 4h, is shown the same progression of events as they would occur in the absence of target. Figure 2a shows the continuous split probe 10 including the 5' end 14 and 3' end 16, as well as the first NCR 22 and the second NCR 24. Similarly, Figure 4a shows the dual SPR 50 including the 5' end 14, the 3' end 16, the first NCR 22 and the second NCR 24. In the absence of target, no ligation junction is formed as is shown in Figures 2b and 4b. If unligated SPR is not separated out, first primer 28 can anneal to its PBS 26 is shown in Figures 2c and 4c and, as shown in Figures 2d and 4d, a partial extension product 66 is formed. But in the absence of a ligation junction the extension product is incomplete as shown in Figures 2d and 4d. Nevertheless,

split probe 10 and split probe half 51 will continue in subsequent cycles to provide a PBS 26 for first primer 28 and multiple incomplete extension products 66 will be formed as is shown in Figures 2e, 2f, 4e and 4f. In the absence of extension across the ligation junction, extension product 66 will never contain the second PBS 34 which would permit hybridization and extension of the second primer 32. Thus, as shown in Figures 2g, 2h, 4g and 4h there will be no second extension product whatsoever and, thus, no exponential amplification.

The great advantage of the present invention is that each split probe reagent can be designed to include 3' and 5' ends which are specific for a particular target, while including in their NCRs two sequences that ensure PBSs which are common to a plurality of SPRs. In this manner, a single set of common primers, and a single set of amplification conditions which are optimized for the common primers, can be used to amplify a plurality of target sequences without sacrificing or compromising the efficiency of amplification. Double-stranded amplification products are generated only in the presence of the specific target of interest, which causes formation of the ligation junction. Accordingly, even under common, simultaneous reaction conditions, a specific SPR will be amplified only if its specific and unique target was present initially to cause ligation of the split probe reagent.

Detection of the target sequence(s) proceeds according to any known method for detecting the amplification products of a PCR. Several possible methods are discussed below, keeping in mind the requirements imposed by multiplex assays. The first consideration is whether or not it is necessary in a particular case to differentiate among the targets detected in a multiplex assay. Depending on the targets, differentiation may not be require. For example, multiplex assays may be performed to detect HIV-1 and HIV-2, but the practitioner may need to know only whether either is present. Similarly, a practitioner may need to know only that a patient is infected with a "high-risk" HPV type, not whether it is type 16, type 18 or type 33. In these cases it is uneccesary to differentiate among the various targets and a generic detection scheme may be used.

In contrast, when the practitioner needs to differentiate the targets, a discriminating detection scheme is required. For example, in genetic testing, it may be necessary to know which of two or more alleles is present. In such cases where discriminating detection is desired in heterogeneous assays, it is preferred to use a capture system and label system such that at least one (capture or label) system is unique for each target and specific for the

amplification product made in the presence of that target. The other system may be common to all targets or may also be unique.

Several methods of unique capture are known and include hybridizatio with an immobilized or immobilizable probe complementary to a unique segment of the SPR (or amplification product therefrom). Such a unique segment may be the target specific regions of the 3' and/or 5' ends or it may be a distinct unique segment inserted into the SPR at any location that is amplified. By using unique capture to physically separate the distinct targets, a common label can be attached to all yet the targets are differentiated on the basis of spatia separation.

Unique labels include labels that can be differentiated, such as on the basis of wavelength (including color). For example, differently colored particle labels can differentiate, as can fluorophores or chemilumiphores of varying wavelength. It may be simpler to perform a unique capture/separation and employ a common label/detection system. For example, beads or microparticles which can be physically separated (eg. by manipulation, filtration, chromatography, sedimentation, centrifugation, magnetic field, etc.) may be used as a solid phase. Separable solid phases are each coated with a specific binding member that, directly or through intermediary specific binding members, is capable of recognizing a unique SPR (or amplification product therefrom). After amplification and incubation with the solid phases, the solid phases are separated and a common label is used. Different target sequences are determined by the appearance of signal on one or more of the different solid phase groups.

Common capture or detection can be accomplished by, for example, including a common detection segment in each SPR. The segment may overlap with the primer regions, since they are common already, but this may be less desirable due to potential interference from primer artifact products. A distinct segment, preferably between the PBS' and the ligation junction, (and downstream from the PBS' sequence) as is shown in Example 1, is preferred. Alternatively, a common capture or detection component can be introduced via the primers. Since the primers are common to all SPRs a common label or specific binding member is easily incorporated into all amplification products. For example, the primers may contain a $^{32}P$ tag or they may be biotinylated or otherwise coupled to specific binding members, preferably at or near their 5' terminus. Techniques for so labeling primers are well known in the art.

From the above discussion of detection, it should be clear that specialized segments can be included in SPRs to facilitate separation and/or detection of

amplification products. For example, it may be desirable to include one or more sequence segments for the isolation or capture of extension products as well as one or more sequence segments for hybridization of a detection probe. Such sequences may be common to all SPRs where discrimination is not needed, but at least one such sequence should be unique where discrimination is desired.

Another specialized insertion may simply include an additional length segment, the sequence of which is unimportant provided it does not contain target specific regions or PBSs. Where the amplification products will be run on a gel, it is possible to differentiate them on the basis of their length (and/or molecular weight) as is well known. For example, a SPR specific for a first target sequence might have 3' and 5' ends of 15 bases each, and first and second NCRs of 20 bases each with the PBSs at the extreme outsides, for a total amplification product length of 70 bases. A SPR for a second target might also have 15 base 3' and 5' ends and a first NCR identical to the first SPR. The second SPR, however, has a 30 base second NCR with the extra 10 bases being upstream of the PBS. This ensures that the amplification products of the second SPR will be 80 bases and differentiable from those of the first SPR. Similarly, a third (and subsequent) SPRs might have 3' and 5' ends specific for individual targets and comparable NCRs with additional insertions, increasing in length for each distinct SPR. This approach is used in Example 2. It is fairly routine to discriminate base pair differences of 10 or more in the range of 50 to 200 total bases. Such gels may be visualized with stains such as ethidium bromide or by employing isotopically labeled primers.

Of course, there are many variations on this technique all of which are within the invention, including the use of mediator capture probes as is disclosed in U.S. Patent 4,751,177 to Stabinsky; and the use of an intermediate detection probe as is disclosed in U.S. Patent 4,882,269 to Schneider, et al. Other variations include virtually any order of combination of the reagents, preferably using isolation on a solid phase as a final step. Those skilled in the art are readily able to devise varying orders of reaction to suit their individual needs, and varying capture/detecti n configurations.

C. *Kits of the Invention*

Kits according to the invention include a suitable supply of the following reagents in one or more suitable containers:

- at least one SPR for each desired target nucleic acid sequence;
- an agent for ligation, preferably a ligase enzyme;

- a first common primer having a sequence complementary to the first common PBS in the first NCR of the SPR; and second common prime having a sequence identical to the second PBS' site in the second NCR of the SPR;
- an agent for inducing extension, such as a polymerase, preferably a thermostable polymerase; and
- deoxynucleotide triphosphates.

Ideally, all reagents may be present in a single unit dose container. More practically, it may be necessary to separate or temporarily inactivate certain reagents. For example, enzyme might be isolated or inactivated by means of elimination of a required cofactor. The kit may optionally include further means for detection, such as capture probes, detection probes, enzyme labels and/or substrates. Additionally, the kit may include means for isolation or separation of ligated probe complexes.

The invention will now be described by some specific examples which serve to illustrate the invention. Reaction conditions, reaction times and temperatures, and number of amplification cycles have been chosen for example only. Optimization of each is of routine practice for one skilled in the art so the full scope of the invention is to be defined by the appended claims.

*D. Examples*

Example 1: Multiplex amplification and detection of DNA from several HPV types using a single primer set.

Each of the following polynucleotides is synthesized:

A HPV 16 specific split probe reagent (SEQ ID NO. 1) having the sequence:

5'TGCATAAGCA CTAGCATTTT CTGTGCTCTT *GTACGGACAC GAACTCATCC* TCT*GGTTATC GAAATCAGCC ACAGCTCTTG* **GAGGAGTCCA TGACGAAACT** CTCTCTATTA TCCACACCTG CATTTGC-3';

A HPV 18 specific split probe reagent (SEQ ID NO. 2) having the sequence:

5'CGTGGCGGCA TGGGAACTTT CAGTGCTCTT *GTACGGACAC GAACTCATCC* TCT*GGTTATC GAAATCAGCC ACAGCTCTTG* **GAGGAGTCCA TGACGAAACT** CTCCCTAACG TCCTCAGAAA CATTAGA-3';

A HPV 33 specific split probe reagent (SEQ ID NO. 3) having the sequence:

5'AGGATACTTG TTACCGGTTT CAGTGCTCTT *GTACGGACAC GAACTCATCC*
*TCTGGTTATC GAAATCAGCC ACAGCTCTTG* **GAGGAGTCCA TGACGAAACT**
CTCCCTATTA TCAGCACCCG GTTGTCC-3';

A common PCR primer A (SEQ ID NO. 4) having the sequence:

5'-GATGAGTTCG TGTCCGTACA-3';

A common PCR primer B (SEQ ID NO. 5) having the sequence:

5'-GGTTATCGAA ATCAGCCACA G-3'; and

A generic detection probe (SEQ ID NO. 6) having the sequence:

5'-TGGAGGAGTC CATGACGAAA-3'

In the three SPRs (SEQ ID NOs 1-3) the underlined portions represent the 5' end and 3' end which are specific for the respective HPV targets. The 5' end of each SPR (SEQ ID NOs 1-3) is phosphorylated to provide a ligatable end. Primer A (SEQ ID NO. 4) is complementary to the first italicized sequence; primer B (SEQ ID NO. 5) is identical to the second italicized sequence since it, like segment 30 (figures 1c and 2c), is a PBS' site. The bold sequence segment is that of the detection probe (SEQ ID NO. 6), which will hybridize to the complementary strand of the SPR. The primer sequences and the detection probe sequence are derived from bacteriophage lambda.

Individual reactions containing either no HPV DNA or various concentrations of HPV 16, HPV 18 or HPV 33 DNA are incubated with each SPR (SEQ ID NOs. 1-3). The reaction contains 10-50 mM Tris pH 8.3, 1-5 mM $MgCl_2$, 20-50 mM KCl, 0.1-10 µM NAD/ATP cofactor, 1-5 units of DNA ligase and approximately 15-30 nM of SPR in a volume of 50 µl. The reactions are heated to 95°C for 1 minute to denature all double stranded nucleic acid, and cooled to 55°C for 1-60 min. to allow the split probes to anneal to their respective HPV targets. When annealed to the proper HPV target sequence, DNA ligase will join the 5' and 3' ends of the split probe to produce a circular molecule. The reactions are then heated to 100°C for 10 min. to inactivate the ligase if desired.

Reactions are then diluted to 100 µl for PCR amplification. Final PCR conditions are 20-50 mM Tris pH 8.3, 20-50 mM KCl, 1-5 mM $MgCl_2$, 200 mM each dATP, dCTP, dGTP, TTP, 0.001% (w/v) gelatin, about 1µM each common PCR primers A and B (SEQ ID NOs. 4-5) and 1-5 units *Taq* DNA polymerase. Reactions are cycled for 40 cycles of consisting of 1 minute at 95°C, 1 min at 55°C and 1 min. at 65°C.

After amplification, reaction products are visualized following electrophoresis through a 1% agarose gel and staining with ethidium bromide. The predicted 127 base pair PCR product is produced only when the proper split probe and HPV target are present in the same reaction.

In an alternative detection method, PCR reaction products are mixed with an excess of the generic detection probe (SEQ ID NO. 6) which has been $^{32}$P-labeled at the 5' end with $\gamma$-$^{32}$P-ATP and polynucleotide kinase. The mixture is heated to 95°C for 1-5 min. and cooled to 55°C for 1-5 min. An aliquot is then electrophoresed through a 10% non-denaturing polyacrylamide gel. Hybridization of the probe to PCR amplification product is identified after autoradiography. Once again, the labeled gel shift products are produced only when the proper split probe and HPV target are present in the same reaction.

Example 2: Multiplex amplification of sections of the CFTR gene and detection of Cystic Fibrosis mutations

Cystic fibrosis ("CF") is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Gene. Zielenski, et al. *Genomic DNA sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene*, Genomics 10, 214-228 (1991). The mutations most commonly causing CF are those specified as $\Delta F_{508}$, $G_{551}D$ and $W_{1282}X$. $\Delta F_{508}$ specifies a three base deletion at DNA position 1653 which causes the loss of the phenylalanine which occurs at position 508 of the normal CFTR protein; $G_{551}D$ specifies a G to A change at DNA position 1784, causing replacement of the normally-occurring glycine at position 551 with aspartic acid; and $W_{1282}X$ specify truncation of the CFTR protein after position 1281 by creating a nonsense mutation (G to A at DNA position 3978) causing the gene to code for no amino acid at position 1282, rather than the normally occurring tryptophan.

Each of the following polynucleotides is synthesized;

A $G_{551}D$ specific split probe reagent (SEQ ID NO. 7) having the sequence:

5'-TCTCCACTCA GTGTGATTCC CTCT*TGTACG GACACGAACT CATCCTCTGG TTATCGAAAT CAGCCACAGC* TCTTGGAGGA GTCCATGACG AAACTCTAAA GAAATTCTTG CTCGTTGA-3';

A $W_{1282}X$ specific split probe reagent (SEQ ID NO. 8) having the sequence:

5'-CACTGTTGCA AAGTTATTGA ATCTCT*TGTA CGGACACGAA CTCATCCTCT GGTTATCGAA ATCAGCCACA* GCTCTTGGAG GAGTCCATGA CGAAAGATGA ACTGATTGCC CGTCTCTCTA CTCCAAAGGC TTTCCTT-3';

A $\Delta F_{508}$ specific split probe reagent (SEQ ID NO. 9) having the sequence:
5'-ATGATATTTT CTTTAATGGT GCCCTCTTGT ACGGACACGA ACTCATCCTC
TGGTTATCGA AATCAGCCAC AGCTCTTGGA GGAGTCCATG ACGAAAGATG
AACTGATTGC CCGTCTCCGC TCGCTGGGTG AACAACCTCT TATATTCATC
ATAGGAAACA CCA-3';

A common PCR primer A (SEQ ID NO. 4) having the sequence:
5'-GATGAGTTCG TGTCCGTACA-3';
A common PCR primer B (SEQ ID NO. 5) having the sequence:
5'-GGTTATCGAA ATCAGCCACA G-3';
A $G_{551}D$ detection probe (SEQ ID NO. 10):
5'-AAAGAAATTC TTGCTCGTTG ATCTCCACTC AGTGTGATTC C-3';
A $W_{1282}X$ detection probe (SEQ ID NO. 11):
5'-ACTCCAAAGG CTTTCCTTCA CTGTTGCAAA GTTATTGAAT-3'; and
A $\Delta F_{508}$ detection probe (SEQ ID NO. 12):
5'-TATATTCATC ATAGGAAACA CCAATGATAT TTTCTTTAAT GGTGCC-3'.

In the three SPRs (SEQ ID NOs 7-9) the underlined portions represent the 5' end and 3' end which are specific for the respective mutations in the CFTR gene. The detection probes (SEQ ID NOs 10-12) are designed to hybridize with these underlined regions once the ends are ligated. The 5' end of each SPR (SEQ ID NOs 7-9) is phosphorylated to provide a ligatable end. Primer A (SEQ ID NO. 4) is complementary to the first italicized sequence and primer B (SEQ ID NO. 5) is identical to the second italicized sequence since it, like segment 30 (figures 1c and 2c), is a PBS' site. The primer sequences and the differential length insertion sequences are derived from bacteriophage lambda

Individual reactions including genomic DNA containing either the $G_{551}D$, the $W_{1282}X$ or the $\Delta F_{508}$ mutation within the CF gene are incubated with each SPR (SEQ ID NOs. 7-9). The reaction contains 10-50 mM Tris pH 8.3, 1-5 mM $MgCl_2$, 20-50 mM KCl, 0.1-10 µM NAD/ATP cofactor, 1-5 units of DNA ligase and approximately 15-30 nM of SPR in a volume of 50 µl. The reactions are heated to 95°C for 1 minute to denature all double stranded nucleic acid, and cooled to 55°C for 1-60 min. to allow the split probes to anneal to their respective mutant targets. When annealed to the proper mutant target sequence, DNA ligase will join the 5' and 3' ends of the split probe to produce a circular molecule. The reactions are then heated to 100°C for 10 min. to inactivate the ligase.

Reactions are then diluted to 100 µl for PCR amplification. Final PCR conditions are 20-50 mM Tris pH 8.3, 20-50 mM KCl, 1-5 mM $MgCl_2$, 200 mM each dATP, dCTP, dGTP, TTP, 0.001% (w/v) gelatin, about 1µM common PCR primers A and B (SEQ ID NOs. 4-5) and 1-5 units *Taq* DNA polymerase. Reactions are cycled for 40 cycles of consisting of 1 minute at 95°C, 1 min at 55°C and 1 min. at 65°C.

After amplification, reaction products are visualized following electrophoresis through a 10% polyacrylamide gel and staining with ethidium bromide. The predicted PCR products of 117bp for the $G_{551}D$ mutation, 137bp for the $W_{1282}X$ mutation and 163bp for the $\Delta F_{508}$ mutation are produced only when the proper split probe and mutant target sequence are present in the same reaction.

In an alternative detection method, PCR reaction products are separated by virtue of a specific hybridization capture and are labeled with a common label. Thus, primer A is biotinylated at its 5' end. Following the ligation reaction (as above), unligated SPRs are degraded using exonuclease VII, which is reported not to act on circularized probes. Amplification proceeds as above, the biotinylated primer being incorporated into extension products of the first primer A. Each of the detection probes (SEQ ID NOs 10-12) is spotted onto three discrete spots in a linear array on a sheet of nitrocellulose and dried. The sheet of nitrocellulose is cut into three strips each strip containing a spot for each detector probe. The three strips are incubated under hybridizing conditions with amplified reaction samples from individual patients as follows: strip 1 with sample from a patient having the $G_{551}D$ mutation, strip 2 with sample from a patient having the $W_{1282}X$ mutation, and strip 3 with sample from a patient having the $\Delta F_{508}$ mutation. After incubation, the strips are gently washed and incubated with a conjugate of anti-biotin:colloidal gold. Following a rinse, the strips are observed for the presence of a reddish-brown color at each of the spots. On each strip, only the spot having the detection probe specific for the patient's mutation should be visible.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: John J. Carrino

(ii) TITLE OF INVENTION: Method of Multiplex Amplification

(iii) NUMBER OF SEQUENCES: 12

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Apple Macintosh IIci
(C) OPERATING SYSTEM: System 7.0.1
(D) SOFTWARE: MS Word

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

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(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 127 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGCATAAGCA CTAGCATTTT CTGTGCTCTT GTACGGACAC GAACTCATCC TCTGGTTATC 60
GAAATCAGCC ACAGCTCTTG GAGGAGTCCA TGACGAAACT CTCTCTATTA TCCACACCTG 120
CATTTGC 127

(2) INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 127 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: synthetic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGTGGCGGCA TGGGAACTTT CAGTGCTCTT GTACGGACAC GAACTCATCC TCTGGTTATC
GAAATCAGCC ACAGCTCTTG GAGGAGTCCA TGACGAAACT CTCCCTAACG TCCTCAGAAA
CATTAGA 1
1

(2) INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 127 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: synthetic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGGATACTTG TTACCGGTTT CAGTGCTCTT GTACGGACAC GAACTCATCC TCTGGTTATC 60
GAAATCAGCC ACAGCTCTTG GAGGAGTCCA TGACGAAACT CTCCCTATTA TCAGCACCCG 120
GTTGTCC 127

(2) INFORMATION FOR SEQ ID NO:4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: synthetic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATGAGTTCG TGTCCGTACA 20

(2) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: synthetic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGTTATCGAA ATCAGCCACA G 21

(2) INFORMATION FOR SEQ ID NO:6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: synthetic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TGGAGGAGTC CATGACGAAA 20

(2) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: synthetic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCTCCACTCA GTGTGATTCC CTCTTGTACG GACACGAACT CATCCTCTGG TTATCGAAAT 60
CAGCCACAGC TCTTGGAGGA GTCCATGACG AAACTCTAAA GAAATTCTTG CTCGTTGA 118

(2) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 137 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: synthetic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CACTGTTGCA AAGTTATTGA ATCTCTTGTA CGGACACGAA CTCATCCTCT GGTTATCGAA 60
ATCAGCCACA GCTCTTGGAG GAGTCCATGA CGAAAGATGA ACTGATTGCC CGTCTCTCTA 120
CTCCAAAGGC TTTCCTT 137

(2) INFORMATION FOR SEQ ID NO:9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 163 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: synthetic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGATATTTT CTTTAATGGT GCCCTCTTGT ACGGACACGA ACTCATCCTC TGGTTATCGA 60
AATCAGCCAC AGCTCTTGGA GGAGTCCATG ACGAAAGATG AACTGATTGC CCGTCTCCGC 120
TCGCTGGGTG AACAACCTCT TATATTCATC ATAGGAAACA CCA 163

(2) INFORMATION FOR SEQ ID NO:10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: synthetic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AAAGAAATTC TTGCTCGTTG ATCTCCACTC AGTGTGATTC C 41

(2) INFORMATION FOR SEQ ID NO:11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: synthetic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACTCCAAAGG CTTTCCTTCA CTGTTGCAAA GTTATTGAAT 40

(2) INFORMATION FOR SEQ ID NO:12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TATATTCATC ATAGGAAACA CCAATGATAT TTTCTTTAAT GGTGCC

What is Claimed is:

1. A method for amplifying a target nucleic acid sequence comprising:

(a) forming a reaction mixture under hybridizing conditions with

(i) a sample suspected of containing a target strand with a target sequence of interest, the target strand being present in single stranded form;

(ii) at least one split probe reagent having a 5′ end complementary to a first segment of the target strand and a 3′ end complementary to a second segment of the target strand, the second segment being sufficiently near the first segment such that 5′ end can be joined to the 3′ end when the split probe reagent is hybridized with the target strand, wherein said 5′ end and said 3′ end are on two distinct polynucleotides or on different ends of one continuous polynucleotide; the split probe reagent further having a first non-complementary region located downstream of the 5′ end and not complementary to the target strand, and a second non-complementary region located upstream of the 3′ end and not complementary to the target strand; and

(iii) an agent for ligating together said 3′ and 5′ ends of the split probe reagent;

(b) ligating together said 3′ and 5′ ends of the split probe reagent while hybridized with the target strand thereby to form a ligated probe having a ligation junction;

(c) separating the ligated probe from the target strand;

(d) treating the reaction mixture under hybridizing conditions with:

(i) an excess of amplification primers wherein a first primer has a sequence complementary to a primer binding site ("PBS") located in said first non-complementary region; and wherein a second primer has a sequence identical to a site ("PBS′ ") located in said second non-complementary region, with the proviso that if said split probe reagent is continuous the PBS located in said first non-complementary region is upstream of said PBS′ located in said second non-complementary region;

(ii) a supply of deoxynucleotide triphosphates; and

(iii) an agent for inducing extension of the primers;

(e) extending the first primer to form an extension product therefrom;

(f) treating the reaction mixture under denaturing conditions to separate primer extension products from their templates;

(g) treating the reaction mixture under hybridizing conditions to anneal the primers to the ligated probe or to extension product of the first primer and extending the primers to form extension products therefrom.

2. The method of claim 1 further comprising a step of detecting the formation of primer extension products that have been extended through the ligation junction.

3. The method of claim 2 wherein said split probe reagent includes:

(a) a further segment having a unique sequence that is recognized by a labeled probe or a capture probe that is unique for a specific target; or

(b) a further segment having a defined length such that the total length of extension product formed therefrom is unique for a specific target sequence.

4. The method of claim1 wherein the split probe reagent comprises one continuous polynucleotide such that, when ligated, a continuous polynucleotide loop is formed.

5. The method of claim 1 further comprising, prior to said separating step of step (c), an additional step of separating split-probe-reagent:target complexes from unhybridized split probe reagents in the reaction mixture.

6. The method of claim 5 wherein said split probe reagent comprises one continuous polynucleotide and wherein said additional separating step comprises selectively degrading unligated split probe reagent.

7. The method of claim 1 wherein said first and second primers have identical sequences.

8. The method of claim 1 wherein a plurality of split probe reagents are used in the same reaction mixture, each split probe reagent having 5' and 3' ends complementary to different target sequences and each having in common the PBS located in said first non-complementary region and the PBS' site located in said second non-complementary region, whereby each such split probe reagent, once ligated, is amplified by a common primer set.

9. A kit for amplifying a target nucleic acid sequence comprising in one or more containers:

(a) at least one split probe reagent having a 5′ end complementary to a first segment of the target strand and a 3′ end complementary to a second segment of the target strand, the second segment being sufficiently near the first segment such that 5′ end can be joined to the 3′ end when the split probe reagent is hybridized with the target strand, wherein said 5′ end and said 3′ end are on two distinct polynucleotides or on different ends of one continuous polynucleotide; the split probe reagent further having a first non-complementary region located downstream of the 5′ end and not complementary to the target strand, and a second non-complementary region located upstream of the 3′ end and not complementary to the target strand;

(b) an agent for ligating together said 3′ and 5′ ends of the split probe reagent;

(c) an excess of primers wherein a first primer has a sequence complementary to a primer binding site ("PBS") located in said first non-complementary region; and wherein a second primer has a sequence identical to a site ("PBS′ ") located in said second non-complementary region, with the proviso that if said split probe reagent is continuous the primer binding site located in said first non-complementary region is upstream of said site located in said second non-complementary region;

(d) a supply of deoxynucleotide triphosphates; and

(e) an agent for inducing extension of the primers.

10. The kit of claim 9 comprising a plurality of split probe reagents, each split probe reagent having 5′ and 3′ ends complementary to a different target sequences and each having in common the PBS located in said first non-complementary region and the PBS′ site located in said second non-complementary region, whereby each such split probe reagent, once ligated, can be amplified by a common primer set.

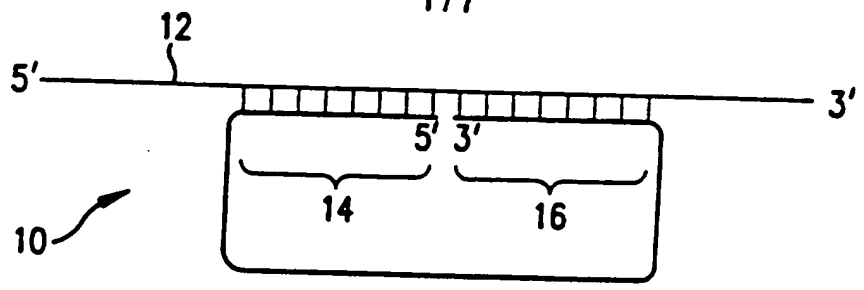


FIG.1a

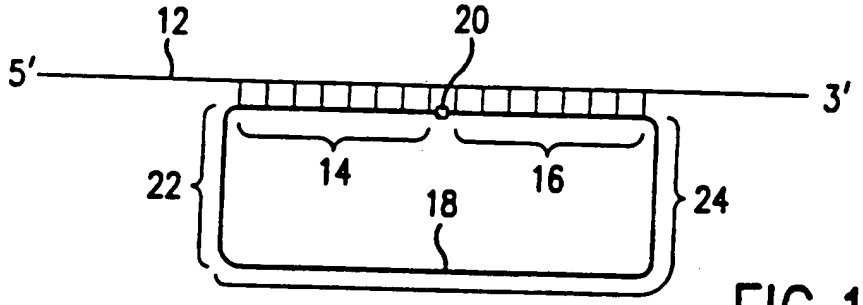


FIG.1b

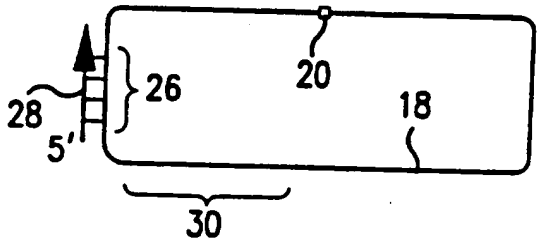


FIG.1c

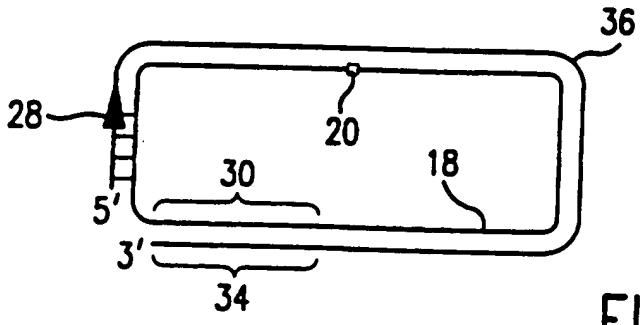


FIG.1d

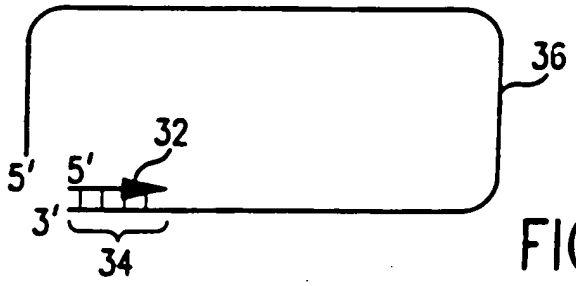


FIG.1e

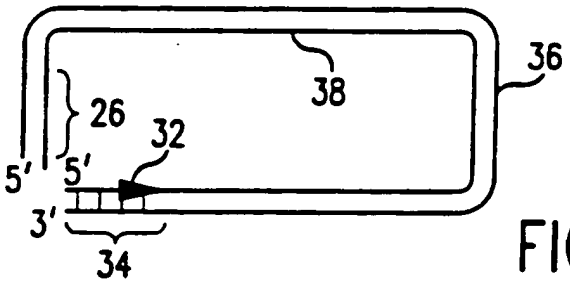


FIG.1f

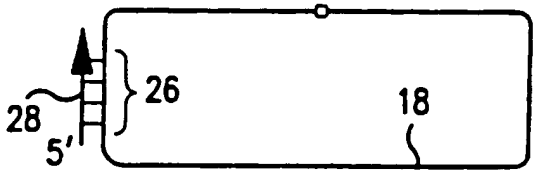


FIG.1g

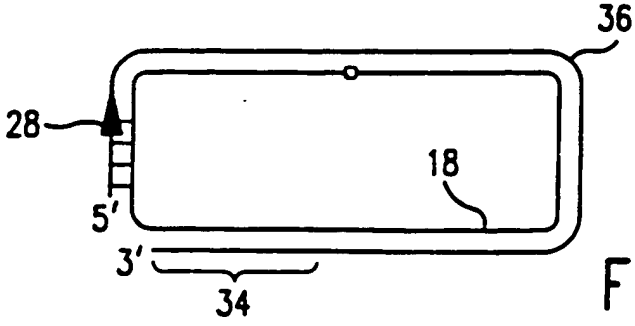


FIG.1h

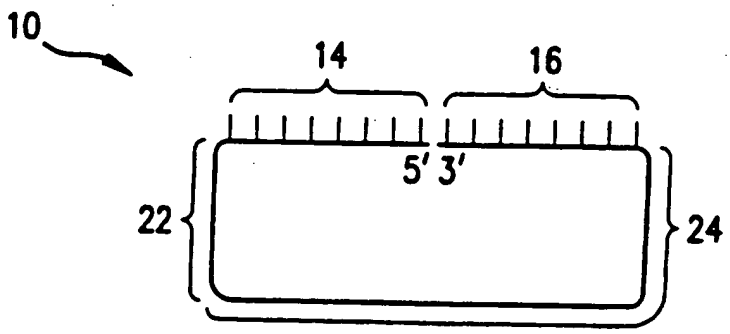


FIG.2a

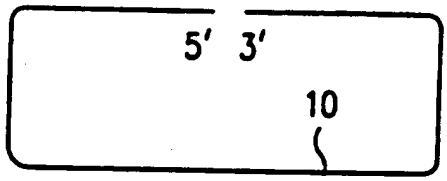


FIG.2b

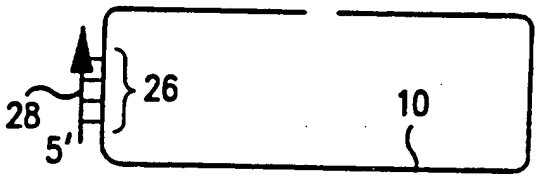


FIG.2c

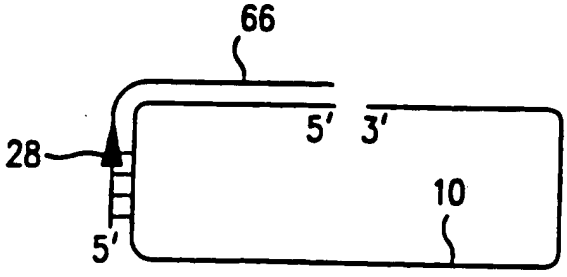


FIG.2d

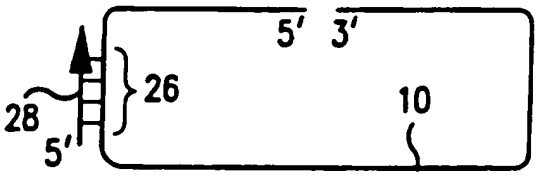


FIG.2e

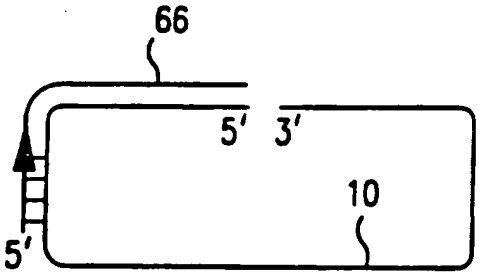


FIG.2f

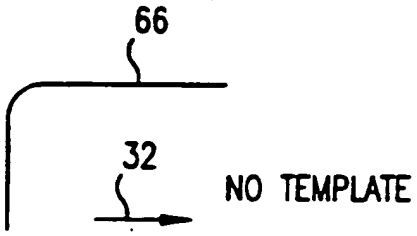


FIG.2g

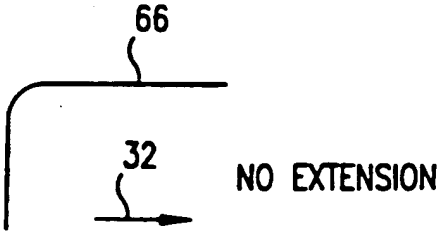


FIG.2h

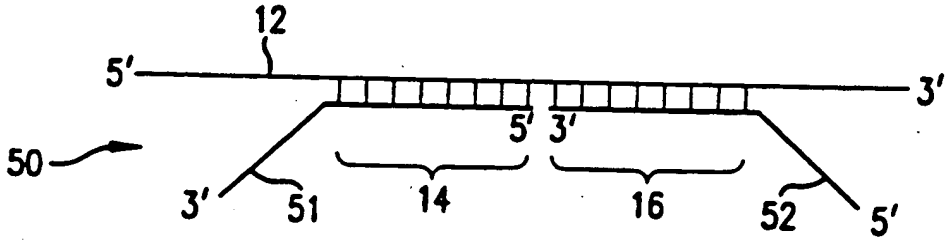


FIG.3a

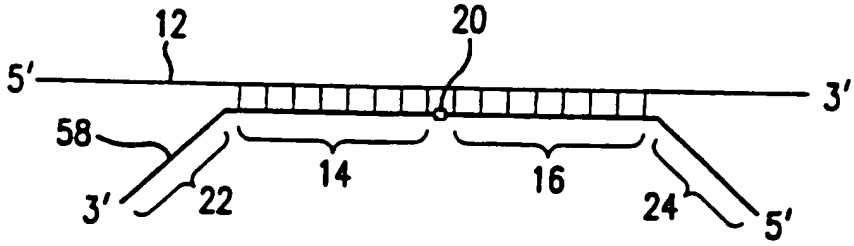


FIG.3b

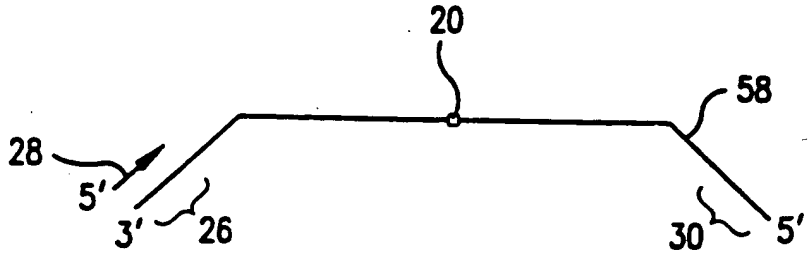


FIG.3c

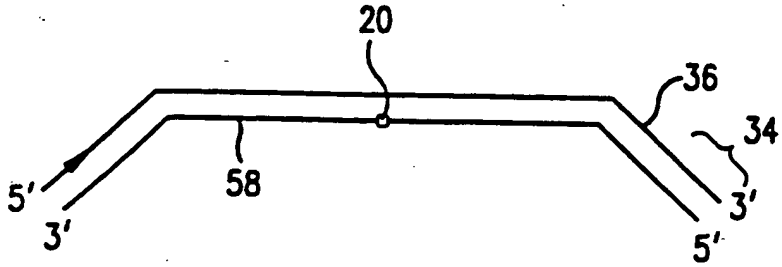


FIG.3d

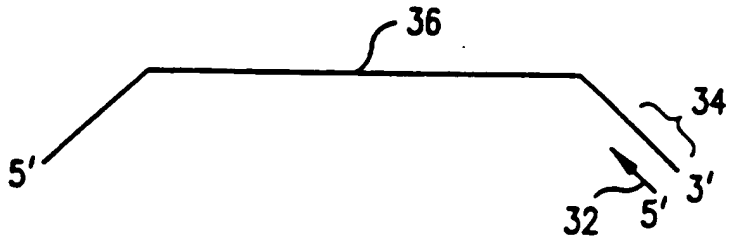


FIG.3e

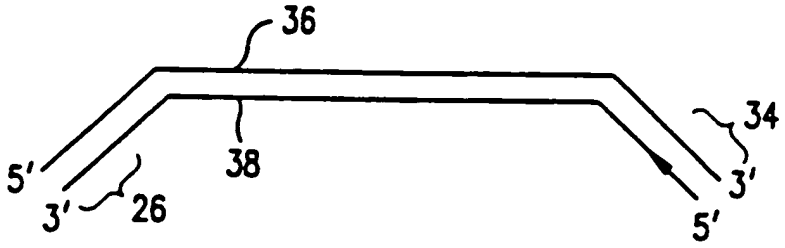


FIG.3f

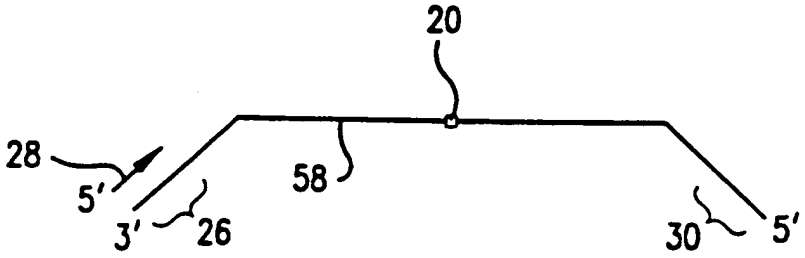


FIG.3g

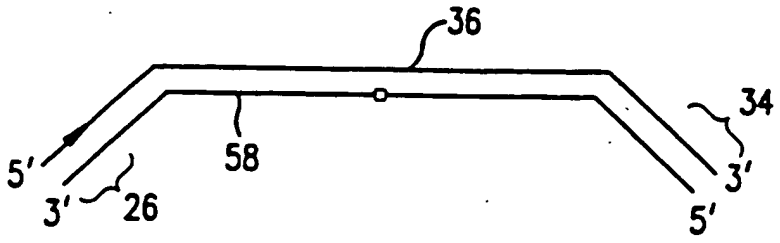


FIG.3h

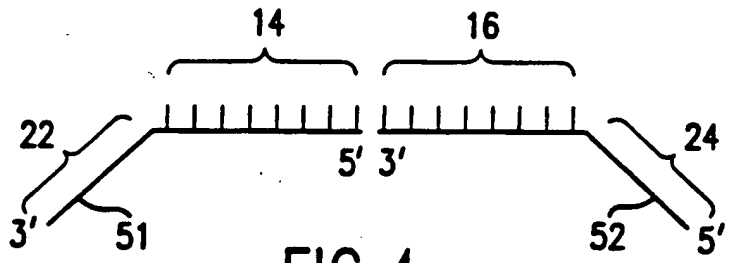


FIG.4a

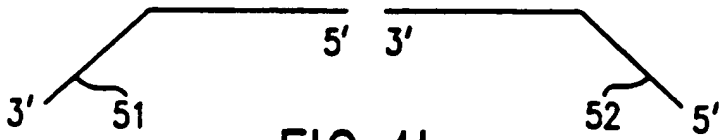


FIG.4b

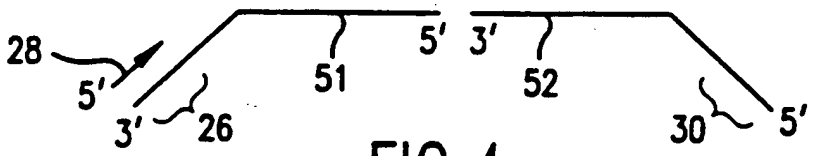


FIG.4c

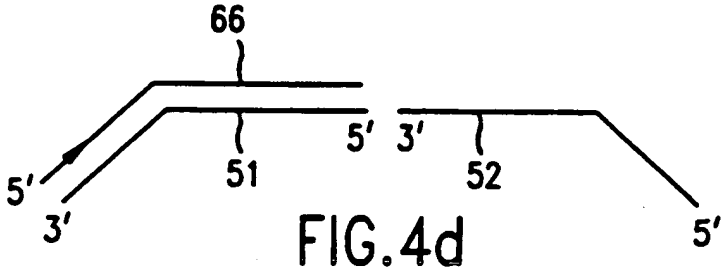


FIG.4d

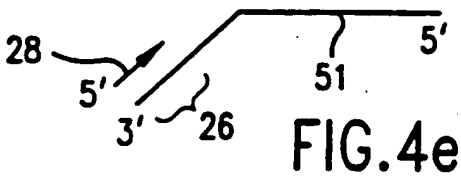


FIG.4e

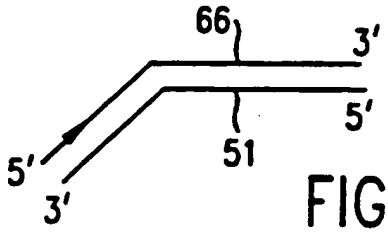


FIG.4f

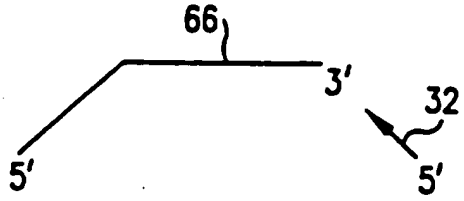


FIG.4g NO TEMPLATE

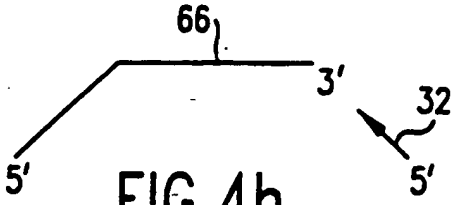


FIG.4h NO EXTENSION

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/14886

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| E | WO,A,95 35390 (MOUNT SINAI SCHOOL OF MEDCINE OF THE CITY UNIVERSITY OF NEW YORK) 28 December 1995<br>see the whole document<br>--- | 1-10 |
| A | SCIENCE,<br>vol. 265, 30 September 1994<br>AAAS,WASHINGTON,DC,US,<br>pages 2085-2088,<br>M. NILSSON ET AL. 'Padlock probes: Circularizing oligonucleotides for localized DNA detection'<br>cited in the application<br>see the whole document<br>---<br>-/-- | 1-10 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 3 April 1996 | 16.04.96 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+ 31-70) 340-3016 | Hornig, H |

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# INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/14886

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | PROC. NATL.ACAD SCI., vol. 87, November 1990 NATL. ACAD SCI.,WASHINGTON,DC,US;, pages 8923-8927, D.A. NICKERSON ET AL. 'Automated DNA diagnostics using an ELISA-based oligonucleotide ligation assay' see the whole document ----- | 1-10 |

1

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/14886

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO-A-9535390 | 28-12-95 | NONE | |